Postimplantation Whole Embryo Culture: A New Method for Studying Ocular Development

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The molecular events involved in normal and abnormal eye development are of pivotal importance, but the living mammalian embryo during organogenesis is virtually inaccessible to the investigator. We describe a method of whole embryo culture applicable both to rat and to mouse. With these techniques, embryos in vitro develop normally through the crucial period of organogenesis, which includes early eye development. This method will enable us to manipulate the milieu in which the embryo develops, and so enable us to study the role of growth factors and other molecules in this important but hitherto inaccessible stage of ocular ontogeny. Invest Ophthalmol Vis Sci 31:1653–1656, 1990

Embryologists have long been fascinated by the growth, induction, and differentiation of embryonal dorsal ectoderm cells, which constitute the earliest events in ocular organogenesis.1 However, in mammals, the inaccessibility of the embryo, its small size, and its reliance on placental nutrition make early ocular development extremely difficult to observe in a continuous fashion. Hsu2 introduced a method that allowed the mouse blastocyst to be cultured through the implantation period, but the survival rate was no more than 50% and development continued only to the early somite stage. In 1964 New and Stein3 developed a culture method for postimplantation rat embryos based on homologous serum; this was far more successful than any used hitherto. Static in vitro techniques, however, produced high rates of embryonic death and less-than-optimal growth rates. Subsequently, New and colleagues produced several methods using roller bottles or circulating serum, which proved decidedly superior. When the homologous serum was immediately centrifuged upon withdrawal and heat-inactivated prior to use, in vitro development approximated that observed in vivo. Current methodology has been reviewed by New4 and in vitro development stages have been classified by Brown and Fabro.5

Materials and Methods. Experimental animals (Wistar rats and BALB/c mice, Leicester University Biomedical Sciences Unit), were treated according to the ARVO Resolution on the Use of Animals in Research. Pregnancy was counted from midnight preceding the morning on which vaginal plugs were observed, after timed matings. On the 8th day after conception for mice (E 8.5), and the 9th day after conception for rats (E 9.5), the pregnant animals were anesthetized with ether; blood was drawn for serum; and the uterus was removed and placed in a Petri dish filled with sterile Hank’s solution. The uterus was divided into segments containing the individual conceptuses; the uterine musculature was torn carefully along the anti-mesenteric border with the use of sterile jeweller’s forceps; and the pear-shaped decidual mass was dissected out. With jeweller’s forceps and a dissecting microscope, the embryo was removed from the center of the decidual mass and examined to determine that the yolk sac was intact.

The parietal yolk sac wall, together with Reichert’s membrane, then was carefully dissected away and the egg-cylinder-stage rat and mouse embryos were placed into heat-inactivated 100% adult rat or 50% rat/50% mouse serum respectively (1 ml per embryo with up to five embryos per bottle), in sterile culture bottles. This serum had been centrifuged immediately after harvesting from the adult animals, and penicillin (5000 IU/ml) and streptomycin (5000 μg/ml) were added. The serum was heat-inactivated at 58°C for 10 min immediately prior to use. The bottles were gassed with 5% oxygen (5% CO₂, 90% N₂) for 2 min, stoppered with a sterile bung, and placed in an incubator on rollers at 37°C. Twenty-four hr later the bottles were gassed with 20% oxygen (5% CO₂, 75% N₂), and about 4 hr before the embryos were harvested the bottles were further gassed with 40% oxygen (5% CO₂, 55% N₂).

After 2 days in culture the yolk sacs of a group of five embryos were opened carefully, with forceps and with care to avoid the yolk sac vessels, to facilitate access of medium and oxygen to the growing embryo. Another group of age-matched embryos had their yolk sacs left intact.

Living embryos were scored daily under a dissecting microscope according to the system described by Brown and Fabro.5 As well as scoring visible features such as yolk sac circulation, we scored brain development, heart development, and somite number, and paid particular attention to the optic system. Scores of eye development that could be judged in the living embryo in culture began with “no sign of optic development,” and then the presence of the sulcus opti-
Egg-cylinder-stage rat embryos (E 9.5) were placed in culture after dissection. e, ectoplacental cone; p, primitive streak embryo; a, amnion. (Bar = 0.5 mm.)

Fig. 1. Egg-cylinder-stage rat embryos (E 9.5) were placed in culture after dissection. e, ectoplacental cone; p, primitive streak embryo; a, amnion. (Bar = 0.5 mm.)

cians, elongated optic primordium, primary optic vesicle with open optic stalk, indented lens plate, and lens pocket or lens vesicle were noted.

After 1, 2, or 3 days in culture (developmental stages E 10.5, 11.5, and 12.5 in the rat, and E 9.5, 10.5, and 11.5 in the mouse), the embryos were fixed in Bouin’s fluid and processed for light microscopy. Embryos were paraffin-embedded and transversely serially sectioned. At each stage, age-matched embryos that had been allowed to develop in utero were processed as normal controls. For each time point reported at least five rat and five mouse embryos were scored, and each experiment was performed at least twice.

The only differences between the rat and mouse experiments were that the murine embryos were grown in 50% mouse/50% rat serum and that the ocular development on days E 10.5 and 11.5 in the mouse was approximately equivalent to E 11.5 and 12.5 in the rat.

Results. At the start of culture, the E 9.5 rat (and the E 8.5 mouse) embryo explant consists of the head fold embryo (three to four somites), together with the extraembryonic membranes (visceral yolk sac, chorion, and the ectoplacental cone) (Fig. 1). After 48 hr in culture, the development of the optic stalk, optic vesicle, and lens ectoderm are comparable in both the cultured embryo (Fig. 2A) and the normal in utero age-matched control (Fig. 2B). In both cases the development of the primary optic vesicle, just prior to its invagination to form the optic cup, may be seen.

One day later, E 12.5 rat embryos that had been in culture for 72 hr and whose yolk sacs had been perforated to allow the embryo to be bathed by medium, showed normal invagination of the optic vesicle and lens vesicle (Fig. 3a). The specialized inner and outer layers of the optic cup were formed normally, and the differentiation of overlying ectoderm to form the lens ectoderm and then lens vesicle occurred in a similar fashion to that seen in the E 12.5 rat embryo that had developed in utero. When the yolk sac surrounding the developing cultured embryo was not breached

Fig. 2. A comparison of the development of the primary optic vesicle in the rat at E 11.5, just prior to its invagination to form the optic cup, after 2 days in vitro (A) and in utero (B). (Bar = 0.2 mm.)

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When mouse embryos were cultured from the same egg cylinder stage (about E 8.5), we again found normal development of the primary optic vesicle and optic stalk in vitro (Fig. 4A), which mirrored the ontogeny observed in utero. This figure shows the beginning of induction between the underlying neural ectoderm and the lens plate. After a further day in culture, the cultured E 11.5 mouse embryos again showed development of the two layers of the optic cup, the lens vesicle, and the separation of the lens vesicle from the overlying ectoderm (Fig. 4B).

**Discussion.** The cellular differentiation that is responsible for the formation of the optic vesicle and then the optic cup begins shortly after gastrulation, approximately 4 days after implantation in the mouse and 1 day later in the rat. By using a method developed to study organogenesis in whole embryo culture, we have shown that it is possible to grow the embryo in vitro during this important developmental period and to achieve growth and differentiation of ocular tissues. This growth and differentiation is similar morphologically, and in a number of functional parameters, to controls that develop in utero up to the formation of the optic cup and lens vesicle.

Although previous authors have studied prenatal rodent eye development, they have been forced to do so by sacrificing entire litters at a preappointed gestational stage. The postimplantation culture system offers the significant advantages that development may be observed in the living animal in culture and...
that ocular development in individual litter members may be serially sampled. Furthermore, recent interest in the role of growth factors in dictating some of the key events in early eye development \(^7\)\(^8\) raises the possibility of manipulating the growth factor content of the medium in which the embryos develop. Such experiments already have yielded significant results with regard to the development of other organs.\(^9\)\(^10\)

Embryos from the one litter can be split into experimental and control culture groups, and the postimplantation culture technique used to explore the effect of surgery or of individual growth factors or other molecules on ocular development. Surgical manipulation and subsequent further culture, or the addition of recombinant growth factors or antibodies either to the medium or directly into the embryo via intravitelline injection, provide exciting potential new tools for studying ocular organogenesis.

**Key words:** eye development, morphogenesis, embryo culture, rat, mouse

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